Volume 218, number 1, 113–118 FEB 04806 June 1987

High extracellular Ca^{2+} and Mg^{2+} stimulate accumulation of inositol phosphates in bovine parathyroid cells

Edward Brown, Peter Enyedi, Meryl LeBoff, Jane Rotberg, Joseph Preston and Chu Chen*

Endocrine-Hypertension Unit, Department of Medicine, Brigham and Women's Hospital and **Endocrinology Division, Department of Medicine, Children's Hospital Medical Center, Boston, MA 02115, USA*

Received 15 April 1987

We examined the effects of the divalent cations Ca^{2+} and Mg^{2+} on inositol phosphate accumulation in bovine parathyroid cells prelabelled with [^{3}H]inositol to determine whether the high extracellular Ca^{2+} and Mg^{2+}-evoked transients in cytosolic Ca^{2+} in these cells might result from increases in cellular IP_3 levels. In the presence of Li^+, both Ca^{2+} and Mg^{2+} produced rapid, 2–6-fold increases in IP_3 and IP_2 and a linear increase in IP of 6–8-fold at 30 min. Smaller (1.5–2-fold) increases in IP_2 and IP_3 were evident within 7.5–15 s upon exposure to high (3 mM) Ca^{2+} in the absence of Li^+. The relative potencies of Ca^{2+} and Mg^{2+} (Ca^{2+} 3-fold more potent than Mg^{2+}) in elevating inositol phosphates were similar to those for their effects in inhibiting PTH release. Fluoride (5 and 10 mM) also produced similar increases in inositol phosphate accumulation, presumably through activation of phospholipase C by a guanine nucleotide (G) protein-dependent process. Thus, high extracellular Ca^{2+} and Mg^{2+}-induced spikes in cytosolic Ca^{2+} in bovine parathyroid cells may be mediated by increases in IP_3, perhaps through a receptor-mediated process linked to phospholipase C by a G-protein.

Inositol phosphate; PTH release; Ca^{2+}; Mg^{2+}

1. INTRODUCTION

In many exocytotic systems, receptor-mediated activation of hormonal secretion is accompanied by increases in the cytosolic Ca^{2+} concentration, which frequently arise both from extracellular as well as intracellular Ca^{2+} [1]. The mobilization of intracellular Ca^{2+} often results from receptor-mediated activation of phospholipase C via a guanine nucleotide (G)-protein, thereby hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). The latter liberates Ca^{2+} from nonmitochondrial stores, predominantly the endoplasmic reticulum [2]. The parathyroid gland is unusual among secretory systems in that increases in the extracellular and cytosolic Ca^{2+} concentrations are associated with inhibition, rather than stimulation, of hormonal secretion [3]. In fura-2-loaded parathyroid cells, Ca^{2+} and Mg^{2+} evoke an initial spike in cytosolic Ca^{2+}, which arises from intracellular Ca^{2+} stores, followed by a sustained increase in the cytosolic Ca^{2+} concentration due to uptake of extracellular Ca^{2+} [4]. These changes in cytosolic Ca^{2+} are analogous to those in other secretory systems and suggest that divalent cation-induced transients in cytosolic Ca^{2+} might arise from generation of cellular IP_3, perhaps resulting from interaction of extracellular divalent cations with a putative cell-surface 'receptor' [4–6]. In the present studies, we have directly examined the effects of extracellular Ca^{2+} and Mg^{2+} on inositol phosphate accumulation in bovine parathyroid cells.

Correspondence address: E.M. Brown, Endocrinology, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)

2. MATERIALS AND METHODS

Bovine parathyroid cells were prepared by digestion with collagenase and DNase using sterile conditions as described [3]. Cells were then cultured in fibronectin-coated cluster wells in medium 199 with 15% neonatal calf serum, penicillin and streptomycin, and 10 mM Hepes, pH 7.45, as outlined [3] for 20–28 h with 30 μCi/ml [^{3}H]inositol (New England Nuclear, Boston, MA). Cells cultured in this fashion routinely showed Ca^{2+}-regulated PTH release which was nearly indistinguishable from that of acutely dispersed cells [3] and fewer than 10% fibroblasts on the light microscope. The cells then were washed free of the labelled precursor with Eagle's MEM ($NaHCO_3$, Ca^{2+}, and Mg^{2+} deleted) containing 0.5 mM Ca^{2+}, 0.5 mM Mg^{2+} and 0.2% (w/v) bovine serum albumin and equilibrated with 1 ml of the same medium for 5–10 min at 37°C. In experiments in which Li^+ was included, 10 mM LiCl was added to the cultured cells 30–60 min prior to washing and was also included in the wash and incubation solutions. At the beginning of the experimental period, the appropriate Ca^{2+} concentration was added to the cells as $CaCl_2$, and the incubation was continued for the desired time interval (7.6 s to 30 min). The reaction was terminated by the addition of a final concentration of 1 M perchloric acid. The cells were then scraped off the culture dish with a rubber policeman and sedimented for 10 min at $1000 \times g$. The supernatant was neutralized with a mixture of 2 N KOH and 1 M Tris base and frozen at −20°C. For determination of [^{3}H]inositol phosphates, the neutralized supernatant was applied to disposable polypropylene columns containing 0.8 ml of

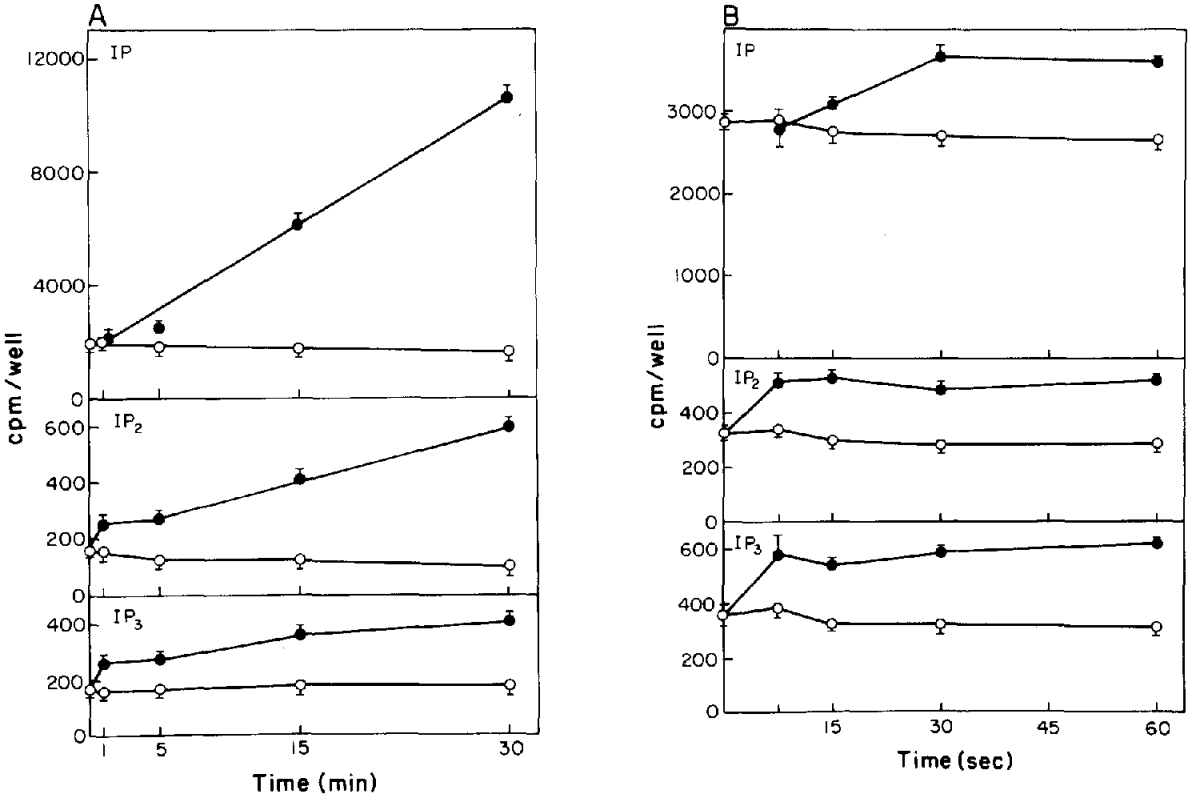

Fig.1. Time course for the stimulation of inositol phosphate accumulation by high Ca^{2+}. After parathyroid cells were cultured with [^{3}H]inositol and were washed free of the labelled precursor, the cells were incubated with either 0.5 mM Ca^{2+} and 0.5 mM Mg^{2+} (open symbols) or 3.0 mM Ca^{2+} and 0.5 mM Mg^{2+} (closed symbols) in the presence (A) or absence (B) of 10 mM Li^+ for the times shown. Results in A are the mean ± SE for 4–6 wells in 2–3 experiments, while those in B are the mean ± SE for three wells from one of three similar experiments.

Dowex AG-1X8 in the formate form and eluted sequentially with distilled water followed by 0.2, 0.4, and 1.0 M ammonium formate in 0.1 M formic acid [7]. PTH in supernatant samples was determined by radioimmunoassay using an antiserum recognizing the intact hormone as well as carboxy-terminal fragments [3]. Statistical comparisons were performed using the unpaired *t*-test.

3. RESULTS

3.1. *Time course for the high Ca^{2+}-induced accumulation of inositol phosphates*

We initially employed medium containing 10 mM Li^+ to assess the effects of high extracellular Ca^{2+} on inositol phosphate accumulation in bovine parathyroid cells, since the recycling of inositol phosphates into phosphoinositides is impaired by the lithium-induced inhibition of the inositol-1-phosphatase. In addition, cultured cells were utilized because preliminary studies revealed that insufficient [^{3}H]inositol was incorporated in short-term experiments (2–4 h) to detect reproducibly changes in IP_2 and IP_3. A high Ca^{2+} concentration maximally suppressing PTH release (3 mM) produced a 1.5–2-fold rise in IP_3 within 1 min, which increased further to 2–3-fold in 30 min, while levels of IP_3 remained constant or declined slightly in cels incubated with a low, maximally stimulatory Ca^{2+} concentration (0.5 mM) (fig.1A). Levels of IP_2 also rose rapidly within 1 min, while the level of IP did not increase significantly at 1 min but rose linearly thereafter to a 6–8-fold increase by 30 min. The temporal sequences of the changes in IP_3, IP_2, and IP are consistent with extracellular Ca^{2+}-induced hydrolysis of polyphosphoinositides with subsequent conversion of IP_3 and IP_2 to IP. In the absence of Li^+, rapid increases in IP_2 and IP_3 were also noted which preceded those in IP (fig.1B).

3.2. *Dose response for the effects of Ca^{2+} and Mg^{2+} on inositol phosphate accumulation*

In the presence of Li^+, IP rose significantly at 1 mM Ca^{2+}, and IP accumulation increased progressively up to 3 mM Ca^{2+} (fig.2). IP_2 and IP_3 showed similar dose response relationships to Ca^{2+}. High Mg^{2+}, which inhibits PTH release with a potency 3-fold less than Ca^{2+} [8,9], also increased inositol phosphate accumulation (fig.2).

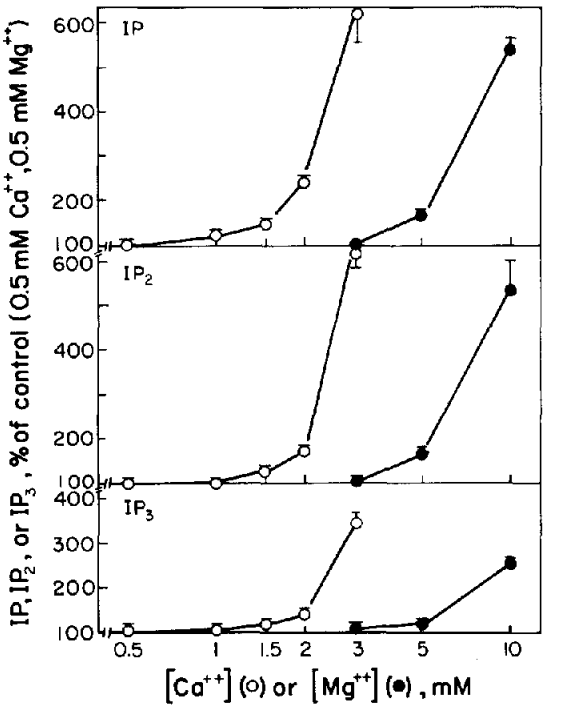

Fig.2. Dose-response for the effects of high extracellular Ca^{2+} and Mg^{2+} on inositol phosphate accumulation. Cells were preincubated with 10 mM Li^+ for 30–60 min, washed, and subsequently incubated with 10 mM Li^+ as well as varying concentrations of Ca^{2+} (○) or Mg^{2+} (●) in the presence of 0.5 mM Mg^{2+} or 0.5 mM Ca^{2+}, respectively. Results show the percent of the value obtained with 0.5 mM Ca^{2+} and 0.5 mM Mg^{2+} in 4–10 wells from 2–5 experiments. The lowest doses of Ca^{2+} increasing IP, IP_2, and IP_3 accumulation significantly were 1.0, 2, and 2 mM, respectively, while for Mg^{2+} the lowest concentration significantly increasing inositol phosphate accumulation was 5 mM in all three cases.

Comparable changes in inositol phosphate levels required about 3-fold higher levels of Mg^{2+} than of Ca^{2+}.

3.3. *Effect of fluoride on inositol phosphate accumulation*

Fluoride ion has been found to activate G-proteins in intact cells and has recently been shown to activate the G-protein mediating phosphoinositide hydrolysis via phospholipase C [10]. Fluoride (5 and 10 mM) increased inositol phosphates in bovine parathyroid cells (fig.3) and also inhibited PTH release to an extent similar to

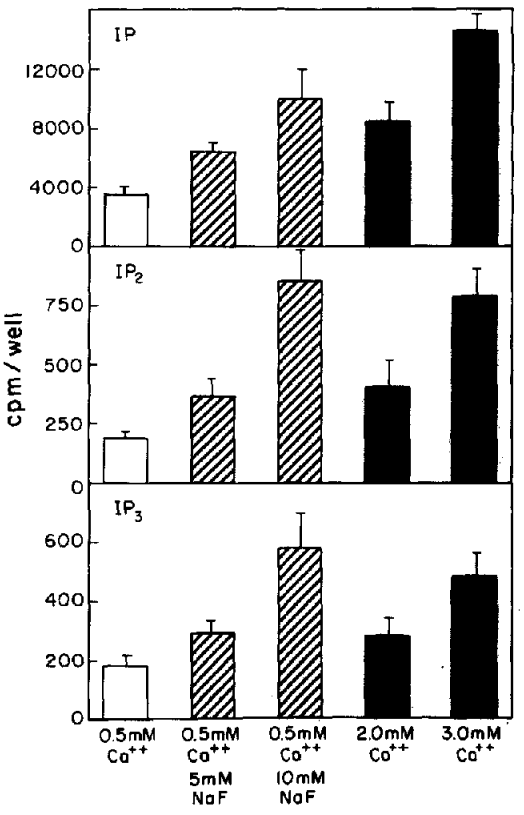

Fig.3. Effect of fluoride on inositol phosphate accumulation. Cells were preincubated for 30-60 min with 10 mM Li^+, washed, and then incubated with 10 mM Li^+, 0.5 mM Mg^{2+} with or without 5 or 10 mM NaF. For comparison, results with 2 or 3 mM Ca^{2+} and 0.5 mM Mg^{2+} are also shown. Results are the mean ± SE for 7 wells in three experiments. Fluoride significantly ($p<0.01$) increased the accumulation of IP and IP_2 at both 5 and 10 mM and IP_3 at 10 mM.

that observed with high Ca^{2+}: PTH release at 0.5 and 2 mM Ca^{2+} were 1.58 ± 0.08 and 0.37 ± 0.05 ng/10^5 cells per 30 min, respectively, while PTH secretion with 0.5 mM Ca^{2+} and 5 mM fluoride was 0.59 ± 0.06 ng/10^5 cells per 30 min.

4. DISCUSSION

The parathyroid gland is unusual among exocytotic systems in that high extracellular Ca^{2+} inhibits, rather than stimulates, PTH release. The effects of Ca^{2+} on parathyroid function are pleiotropic, including not only inhibition of secretion but also reduction in the levels of mRNA for PTH and inhibition of parathyroid cellular proliferation, as well as modulation of several putative mediators, including cAMP, membrane potential, PI turnover, and the cytosolic Ca^{2+} (review [6]). Other divalent cations produce similar effects on parathyroid function, including Mg^{2+}, which is about 3-fold less potent that Ca^{2+} on a molar basis [9]. The mechanisms underlying these diverse actions of polyvalent cations on parathyroid function, however, have remained obscure.

Recent data have suggested that Ca^{2+} and other polyvalent cations exert their effects on the parathyroid gland via a cell surface receptor for these ions. The divalent cation-induced depolarization of rat parathyroid cells could not be explained simply by a change in electromotive force across the plasma membrane, making it necessary to postulate the existence of a cell-surface receptor regulating membrane potential [5]. Furthermore, the increase in cytosolic Ca^{2+} associated with elevations in extracellular Ca^{2+} are reminiscent of those produced by a variety of secretagogues interacting with cell-surface receptors in other types of cells [1]. That is, Ca^{2+} produces an initial 'spike' in cytosolic Ca^{2+}, which arises from intracellular Ca^{2+} stores, followed by a sustained increase in cytosolic Ca^{2+} which derives from uptake of extracellular Ca^{2+} [4,6]. Finally a variety of factors binding to the parathyroid cell surface, such as lectins [11], naturally occurring autoantibodies [12], and monoclonal antibodies [13] modulate PTH release [11-13] and the cytosolic calcium concentration [13], consistent with a role for cell-surface moeities in regulating parathyroid function.

The present results further suggest that extracellular divalent cations modulate parathyroid function, at least in part, through a cell-surface receptor or 'sensor'. Ca^{2+} and Mg^{2+} produce rapid increases in IP_3 which may account for the Ca^{2+} or Mg^{2+}-induced spikes in cytosolic Ca^{2+} via release of cellular Ca^{2+} [14] and which occur with a potency which parallels the relative effectiveness of Ca^{2+} and Mg^{2+} in inhibiting PTH release. It is of interest that these ions continue to raise inositol phosphate content at concentrations which already maximally suppress PTH release, although such discrepancies (e.g. 'spare receptors') are common in the regulation of cellular function by second

messengers. Moreover, lithium, which was included in these dose response studies, has been shown to reduce the sensitivity of PTH release to suppression by high extracellular Ca^{2+} [15]. The temporal relationship of the divalent cation-induced changes in IP, IP_2, and IP_3 are consistent with phospholipase C-mediated hydrolysis of polyphosphoinositides, particularly PIP_2. Furthermore, fluoride ion, which has been found to activate the G-protein mediating activation of phospholipase C in other cells [10], produces changes in inositol phosphates in parathyroid cells which are similar to those brought about by high extracellular Ca^{2+} and Mg^{2+}. By analogy with such other cells, therefore, extracellular divalent cations may bind to a cell-surface receptor which is coupled to phospholipase C via a G-protein.

In addition to changes in IP_3, calcium-induced changes in other second messengers might also be mediated by a receptor coupled to one or more G-proteins. Although the lowering of intracellular cAMP content by high extracellular Ca^{2+} [16] could be related to concomitant changes in cytosolic Ca^{2+}, secondarily inhibiting adenylate cyclase or activating phosphodiesterase, activation of G_i by a putative Ca^{2+} receptor could also reduce cellular cAMP. Similarly, G-proteins have also been found recently to modulate potassium [17] and Ca^{2+} channels [18], thereby providing potential mechanisms for Ca^{2+}-induced changes in membrane potential [5] and in cytosolic Ca^{2+} due to uptake of extracellular Ca^{2+} [4,6], respectively. It is also possible that the sustained increases in cytosolic Ca^{2+} at high extracellular Ca^{2+} might result from alterations in PI metabolism, since IP_4 (inositol 1,3,4,5-tetrakisphosphate) has recently been implicated in mediating uptake of extracellular Ca^{2+} [19].

We employed cultured cells in these studies to achieve sufficient incorporation of [^{3}H]inositol to label the pool of polyphosphoinositides yielding IP_3 and IP_2. Although cells were preincubated for 20–28 h with [^{3}H]inositol, it is possible that complete isotopic equilibrium was not achieved. Thus, changes in [^{3}H]inositol phosphate accumulation may not have provided a totally accurate reflection of changes in the mass of these compounds. Moreover, it is possible that the IP_3 measured in this study may represent a mixture of inositol 1,4,5- and 1,3,4-trisphosphate. Nevertheless, the rapidity and temporal sequence of the appearance of IP_3, IP_2 and IP suggest hydrolysis of phosphoinositides, and inositol 1,3,4,-trisphosphate is thought to arise from this process via IP_4 [20].

In contrast to other secretory cells, activation of PI hydrolysis in the parathyroid cell by divalent cations or fluoride is associated with inhibition, rather than of stimulation, of PTH release. This relationship is particularly intriguing, since in parathyroid cells, as in other cells, agents activating protein kinase C, such as the phorbol ester TPA, stimulate hormone secretion [21]. Moreover, Morrissey has reported that high extracellular Ca^{2+} concentrations lower cellular diacylglycerol (DG) levels [22], rather than raising them as might be expected from activation of phosphoinositide cleavage. Presumably, other mechanisms are responsible for any divalent cation-induced lowering of cellular DG, and these may ultimately determine the secretory response to high extracellular divalent cation concentrations. It is also conceivable that a divalent cation receptor could be directly linked to the exocytotic process via an inhibitory mechanism, without any obligatory linkage to intracellular second messengers.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the expert secretarial assistance of Connie Chisari and Joan Munroe. These studies were supported by USPHS grants AM36796 and AM36801 and grant no. PDT230 from the American Cancer Society.

NOTE ADDED IN PROOF

Shoback [23] has recently reported in abstract form that high extracellular Ca^{2+} enhances accumulation of IP_1 in parathyroid cells.

REFERENCES

[1] Douglas, W.W. (1978) Ciba Foundation Symposium 54, 61–90.
[2] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[3] LeBoff, M.S., Shoback, D., Brown, E.M., Thatcher, J., Lembruno, R., Beaudoin, D., Henry, M., Wilson, R., Pallotta, J., Marynick, S., Stock, J. and Leight, G. (1985) J. Clin. Invest. 75, 49–57.

[4] Nemeth, E.F. and Scarpa, A. (1986) FEBS Lett. 203, 15–19.
[5] Lopez-Barneo, J. and Armstrong, C.M. (1983) J. Gen. Physiol. 82, 269–294.
[6] Brown, E.M., LeBoff, M.S., Oetting, M., Posillico, J.T. and Chen, C. (1987) Recent Prog. Hormone Res. 43, in press.
[7] Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 473–482.
[8] Wallace, J. and Scarpa, A. (1982) J. Biol. Chem. 257, 10613–10616.
[9] Habener, J.F. and Potts, J.T. jr (1976) Endocrinology 98, 197–202.
[10] Blackmore, P.F., Bocckino S.B., Waynick, L.E. and Exton, J.H. (1985) J. Biol. Chem. 260, 14477–14483.
[11] Ota, K., Porat, A. and Sherwood, L.M. (1980) The Endocrine Society 62nd Annual Meeting: Abstr. no. 450, p.187.
[12] Posillico, J.T., Wortsman, J., Srikanta, S., Eisenbarth, G.S., Mallette, L.E. and Brown, E.M. (1986) J. Bone Mineral Res. 1, 475–483.
[13] Posillico, J.T., Srikanta, S., Eisenbarth, G., Quaranta, V., Kajiji, S. and Brown, E.M. (1987) J. Clin. Endocrinol. Metab. 64, 43–50.
[14] Epstein, P.A., Prentki, M. and Attie, M.F. (1985) FEBS Lett. 188, 141–144.
[15] Brown, E.M. (1981) J. Clin. Endocrinol. Metab. 2, 1046–1048.
[16] Brown, E.M., Gardner, D.G., Windeck, R.A. and Aurbach, G.D. (1978) Endocrinology 103, 2323–2333.
[17] Yantani, A., Codina, J., Brown, A.M. and Birnbaumer, L. (1987) Science 235, 207–211.
[18] Hescheler, J., Rosenthal, W., Trautwein, W. and Schultz, G. (1987) Nature 325, 445–447.
[19] Batty, I.R., Nahorski, S.R. and Irvine, R.F. (1985) Biochem. J. 232, 211–215.
[20] Hawkins, P.T., Stephens, L. and Downes, C.P. (1986) Biochem. J. 238, 507–516.
[21] Brown, E.M., Redgrave, J. and Thatcher, J. (1984) FEBS Lett. 175, 72–75.
[22] Morrissey, J.J. (1985) Clin. Res. 33, 440A.
[23] Shoback, D. (1987) J. Bone Mineral Res. 1, 314.